Hepatitis B virus X protein enhances androgen receptor-responsive gene expression depending on androgen level

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Persistent hepatitis B virus (HBV) infection is a major risk of hepatocellular carcinoma (HCC). One intriguing feature of HBV-related HCC is the male predominance, with a male to female ratio of 5–7:1. This dominance has been attributed to the elevated androgen level and the enhanced androgen receptor (AR)-mediated activity in the host. How HBV infection and AR signaling modulate HCC is unknown. We investigated whether the HBV nonstructural protein, X protein (HBx) could cooperate with the AR signaling pathway to enhance carcinogenesis. We found that HBx increased the anchorage-independent colony-formation potency of AR in a nontransformed mouse hepatocyte cell line. We also found that HBx functioned as a positive transcriptional coregulator to increase AR-mediated transcriptional activity. This transcription enhancement was increased in the presence of androgen in a concentration-responsive manner, thus explaining a more prominent effect in males. HBx did not physically associate with ligand-bound AR in the nucleus, and it likely augmented AR activity by increasing the phosphorylation of AR through HBx-mediated activation of the c-Src kinase signaling pathway. Our study documents HBx as a previously undescribed class of noncellular positive coregulators for AR. The results reveal a mechanism for the vulnerability of males to microbial infections and the subsequent development of cancer.

androgen-responsive element

Hepatocellular carcinoma (HCC) is one of the leading cancers in the world, with more than half a million deaths per year (1). Chronic hepatitis, caused by hepatitis B virus (HBV) or hepatitis C virus (HCV) infection, is a major risk factor for the development of HCC (2). Another risk factor is the male gender. HCC occurs much more frequently in men than in women, with a male to female ratio ranging from 2:1 to 11:1 (3). Both animal and human studies support the importance of androgen signaling in determining the male preference of HCC (4–13). It was also noted that the expression and activation of androgen receptor (AR) is increased in the tumor tissues as well as in the surrounding nontumorous liver tissue of patients with HCC (14–16). Intriguingly, the gender preference of HCC differs between HBV- and HCV-related cases. The male predominance in HBV-related HCC is significantly higher than that of HCV-related HCC, with a ratio of 5–7:1 vs. 2–3:1 (17, 18). Among male HBV carriers, those with a higher level of serum androgen and more active AR gene alleles have a significantly increased risk of HCC (19, 20). However, these two factors have not been reported to contribute to the increased risk of HCV-related HCC. Thus, HBV infection might uniquely cooperate with androgen signaling to accelerate hepatocarcinogenesis, raising the possibility that certain HBV gene(s) could modulate AR signaling activity and HCC development.

AR is a ligand-dependent transcription factor that belongs to the nuclear receptor superfamily (21, 22). It consists of several functional domains individually responsible for the activities of ligand binding, DNA binding, nuclear localization, and transcriptional activation. AR-mediated transcription is subjected to many regulations. It occurs by recruiting a variety of cellular coregulators to modulate AR transcriptional activity and to affect the expression of androgen-responsive genes in a cell-type-specific manner (21, 22). Accordingly, we hypothesized that specific HBV gene product(s) may behave as a virus-encoding AR coregulator to enhance AR-responsive gene transcription.

In search of HBV viral factor(s) able to affect androgen-response element-directed transcription, the HBV X protein (HBx) is the most likely candidate. It is the only HBV nonstructural gene that functions as a multifunctional regulator modulating gene transcription, cell responses to genotoxic stress, protein degradation, apoptosis, and several signaling pathways (23, 24). HBx is located in the cytoplasm, with similar distribution patterns as AR in the absence of androgen ligand stimulation. More importantly, some lineages of HBx-transgenic mice develop liver cancers predominantly in the males (6, 25). These observations strengthen the linkage of HBx to male HCC and suggest possible cooperation between HBx and AR activity.

In our present study, we show the carcinogenic potential of HBx-mediated AR activation by its enhancement of the colony-formation capacity of AR in the soft agar transformation assay. We also demonstrate that HBx is a positive transcriptional coregulator of AR and functions in an androgen concentration-dependent manner. To elucidate the mechanism, we found an association of HBx and AR in the cytoplasm. However, in the presence of androgen, the majority of AR translocates into the nucleus to carry out its transcription activity without associating with HBx. The androgen-stimulated phosphorylation of AR increases in the presence of HBx, which is mediated by HBx activation of the c-Src kinase signaling pathway. Therefore, our study supports the role of HBx as a positive coregulator in androgen signaling and provides an explanation for the male predominance of HCC in HBV-infected individuals.

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Abbreviations: AR, androgen receptor; HBV, hepatitis B virus; HBx, HBV X protein; HCV, hepatitis C virus; HCC, heptocellular carcinoma; DHT, dihydrotestosterone.

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Table 1. Anchorage-independent colony-formation assay of parental AML12- and three AML12-derived AR stable transfectant cell lines

Cell line	Transfected plasmid	No. of colonies 5nM R1881 −	+
AML12-vector	pCDNA3	1	3
AML12-vector	pCMV-HBx	0	4
AML12-AR(3D)	pCDNA3	10	20
AML12-AR(3D)	pCMV-HBx	9	41
AML12-AR(3C)	pCDNA3	11	16
AML12-AR(3C)	pCMV-HBx	8	30
AML12-AR(2D)	pCDNA3	6	13
AML12-AR(2D)	pCMV-HBx	8	36

AML12-AR(3D), -AR(3C), and -AR(2D) are independent stable AR transfectants derived from AML12. The cells were transiently transfected with either the pCDNA3 (as vector control) or the pCMV-HBx plasmids and seeded on the soft-agar plates. After 3–4 weeks, colonies >100 μm in diameter from five randomly selected fields were counted. The results shown are average values of two independent experiments.

Results

HBx Increases the Anchorage-Independent Colony-Forming Activity of AR in AML12 Hepatocytes. To evaluate any cooperative role of HBx and AR in hepatocarcinogenesis, we assayed their effect on anchorage-independent colony growth using the mouse AML12 cell line, which is a nontransformed hepatocyte derived from normal mouse liver (26). Because AR is not expressed in AML12 cells, we first established AR transfectants that stably expressed AR. Three independent lines of AR stable transfectants, as well as the control AML12 cells stably transfected with the pSG5-puro vector plasmid, were then transfected with HBx and assayed for their anchorage-independent colony-forming ability in the presence of AR agonist R1881. The results are summarized in Table 1. Only a few colonies (0–4) were detected in control AML12 cells (even when transfected with the HBx construct), and the colony number was significantly increased in the AR stable transfectants upon R1881 treatment (mean, 16 ± 4). Transfection of HBx in these AR-expressing cells further increased the colony number 2- to 3-fold (mean = 36 ± 5). The results suggest that HBx could increase AR-mediated cell transformation activity in a hormone-dependent way.

HBx Enhances AR-Responsive Gene Expression in an Androgen Concentration-Dependent Manner. To elucidate the mechanism by which HBx enhances cell transformation, we first studied the possibility that HBx enhances AR-mediated transcriptional activity. A reporter gene construct, pMMTV-Luc, that contains a luciferase gene linked to androgen-response elements from MMTV was used. It was transfected into HepG2 cells together with the AR and HBx expression plasmids. The cells were then treated with various concentrations of dihydrotestosterone (DHT; Fig. 1*A*, lanes 1–7). Androgen treatment stimulated the cells transfected with AR alone with a 20- to 60-fold increase of luciferase activity (Fig. 1, lanes 2–7, gray bars). However, in cells transfected with HBx-expressing plasmid alone, luciferase activity was not increased (Fig. 1*A*, lanes 2–7, dashed bars). It indicates that HBx alone was incapable of reporter gene activation, and thus HBx does not have ligand-independent AR activation activity. Interestingly, in the presence of DHT (0.05–10 nM), cotransfection of AR and HBx increased reporter activity 3- to 6-fold in comparison with that of cells transfected with AR alone (Fig. 1*A*, lanes 2–7, black bars). Treatment with the AR agonist R1881 can cause coactivation similar to that produced by DHT treatment, and the enhancement of AR

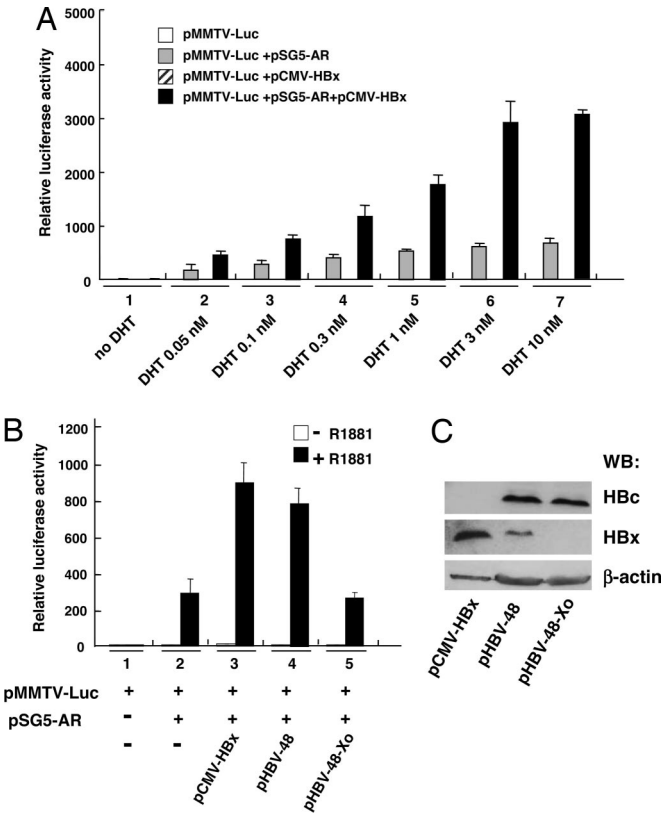

Fig. 1. HBx augments androgen-dependent AR-responsive gene expression. (*A*) HepG2 cells were transfected with pMMTV-luc reporter, pRL-CMV, pSG5-AR, and pCMV-HBx with different combinations. Cells were then treated with DHT with concentrations as indicated, and the cell lysates were prepared for assessment of luciferase activity. The results shown are the average of three experiments (mean ± SD). (*B*) The experiment was similar to the above, except that Huh-7 cells were used, pCMV-HBx was replaced with either pHBV-48 or pHBV-48-X_0, and DHT was replaced with 10 nM R1881. The results shown are the average of three experiments (mean ± SD). (*C*) Western blot analysis was used to verify protein expression by probing with antibodies as indicated. HBc, hepatitis B core antigen.

transcription by HBx was also observed in Huh-7, Hep3B, AML12, and MCF7 cell lines (data not shown).

One important aim of this study was to explain the different carcinogenic consequences of HBV infection in adult males vs. females. Because men and women possess distinct levels of free androgen in serum (27, 28), we thus studied the effects of DHT concentrations on HBx-mediated AR transcriptional activity. Six concentrations of DHT (0.05–10 nM) were tested, corresponding to the serum DHT levels of juveniles (0.05–0.1 nM), adult females (0.1–0.3 nM), and adult males (1–3 nM for most cases and 10 nM as control of extremely high level), respectively (27, 28). Reporter gene activation in cells transfected with AR alone did not show significant increase upon treatment with DHT concentrations >1 nM (Fig. 1*A*, lanes 2–7, gray bars). However, in the presence of HBx, the level of AR transcriptional activity continued to rise with increased DHT concentrations (Fig. 1*A*, lanes 2–7, black bars). The concentration of DHT corresponding to the level of adult males (1–3 nM) gave the highest activation level, compared with those of juveniles and adult females. Therefore, in HBV carriers, AR activation is likely much higher in the infected hepatocytes of adult males than those in the adult females.

To confirm the physiological importance of this observation, we needed to show this AR-enhancement effect in the context of an intact HBV genome instead of only with an isolated

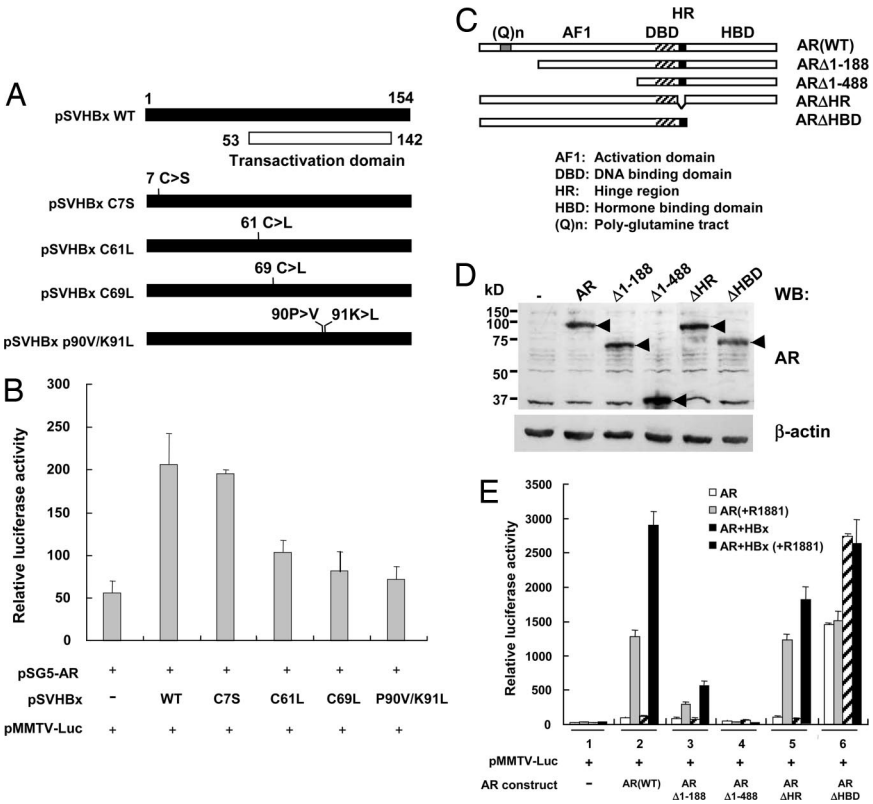

Fig. 2. Functional characterization of HBx and AR regions responsible for HBx-induced AR activation. (*A*) Schematic representation of the four site-directed mutants of HBx used for transfection experiments. (*B*) Similar to Fig. 1*A*, except that pSG5-AR was cotransfected with wild-type or site-directed mutants of pSV-HBx, and 10 nM R1881 was added. The results shown are the average of three experiments (mean ± SD). (*C*) Schematic illustration of wild type and the four deletion constructs of AR used for the transfection experiments with the functional domains and the deletion regions indicated. (*D*) The expression of AR constructs in transfected Huh-7 cells was analyzed by immunoblotting. Protein bands corresponding to AR constructs are indicated by the arrowheads. (*E*) Huh-7 cells were transfected with plasmids as indicated, either treated or untreated with 10 nM R1881, and luciferase activities were determined. Results are the average of three experiments (mean ± SD).

HBx-expression plasmid. For this purpose, we conducted the same AR transcription activation experiments but replaced the HBx expression plasmid with a 1.5-mer HBV genome-containing plasmid, pHBV-48, which can replicate in Huh-7 cells (29). The pMMTV-luc reporter could also be induced by this HBV construct in the presence of AR and R1881 to an extent equivalent to that induced by HBx plasmid alone (Fig. 1*B*, lanes 3 and 4). To exclude the role of other viral genes, we constructed a HBV mutant (pHBV-48-Xo), in which one nucleotide in the HBx ORF was replaced to introduce a premature termination codon. This mutant construct expressed similar levels of HBc and HBs protein as the wild-type construct but failed to express HBx (Fig. 1*C*, lanes 2 and 3). AR transcriptional enhancement was not observed after transfecting pHBV-48-Xo (Fig. 1*B*, lanes 4 and 5). Therefore, the activation of this AR-responsive gene was verified by using the replication-competent HBV genome and suggested the effect could also occur in natural viral infection of human hepatocytes. Moreover, the HBx gene knock-out experiment confirmed that the AR activation effect was strictly through the viral HBx.

Characterization of the Functional Domains of HBx and AR Responsible for HBx-Induced AR Activation. To further characterize the effects of the functional domains of HBx on AR-responsive gene activation, we used several HBx mutant constructs containing specific point mutations. These mutants were designed to remove the cysteine, proline, or lysine residues (Fig. 2*A*) that are conserved among all hepadnaviral X proteins and believed to contribute to HBx transactivation function (30). Except for the C7S mutant, the C61L, C69L, and P90V/K91L HBx mutants all showed diminished enhancement of AR reporter activity (Fig. 2*B*). Wild-type HBx, especially the intact transactivation domain, was thus implicated as being essential for the enhancement of AR activity. In addition, we tested several HBx deletion constructs with deletions located either at the N or C terminus of HBx. All also had reduced reporter gene activity ranging from 50% to 80% (data not shown). Therefore, the intact form of HBx is critical for its effect on AR gene transactivation.

On the other hand, we also tried to map the functional domains of AR responsible for the HBx-mediated transcriptional activation. Four AR deletion constructs were used, including ARΔ1–188, ARΔ1–488, ARΔHR, and ARΔHBD. Each corresponded to constructs lacking the N-terminal polyglutamine tract, the transactivation domain, the hinge region, and the ligand-binding domain, respectively (Fig. 2*C*; ref. 31). The four mutant AR proteins were expressed equivalently after transfecting into Huh-7 cells (Fig. 2*D*).

For cells transfected with the mutant AR constructs alone, the ARΔ1–488 construct had no transcriptional activity, and the second mutant, ARΔ1–188, had impaired activity. Thus, the results were not informative for the subsequent HBx-cotransfection study (Fig. 2*E*, lanes 3 and 4). The third mutant, ARΔHR, maintained transcriptional activity in a ligand-dependent manner, and the HBx-induced activation effect was noted as significantly decreased (Fig. 2*E*, lane 5). The fourth mutant, ARΔHBD, maintained the transcriptional activity in a ligand-independent manner but still retained the HBx-mediated enhancement of AR activity (Fig. 2*E*, lane 6). Because HBx-enhanced AR gene transactivation was

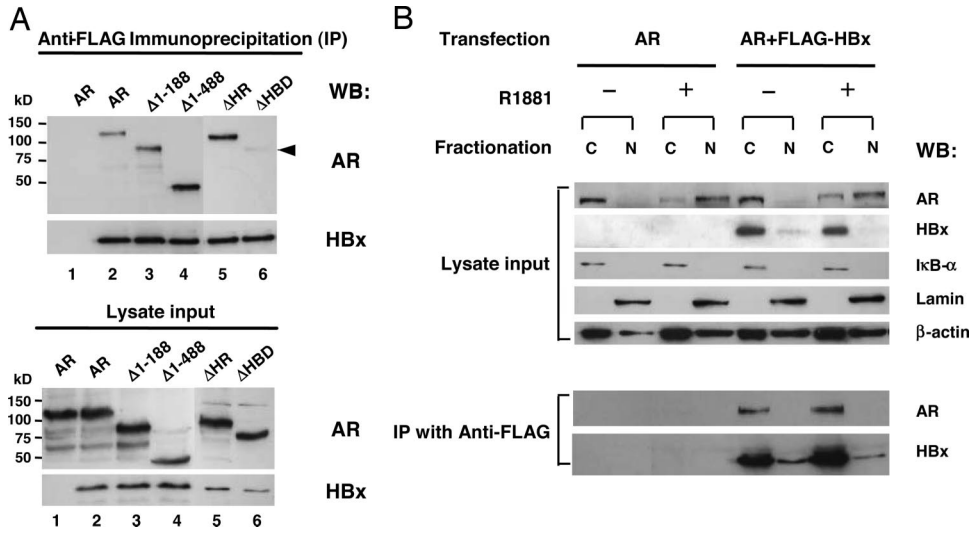

Fig. 3. Characterization of the interaction between AR and HBx and their subcellular distribution pattern in Huh-7 cells. (*A Upper*) Cell lysates from Huh-7 cells transfected with FLAG-HBx and the AR constructs were processed for coimmunoprecipitation analysis, and the immunoprecipitates were analyzed by Western blot analysis probed with AR and HBx antibodies. The position of ARΔHBD protein is denoted by the arrowhead. (*Lower*) Expression of HBx and AR proteins. (*B*) Huh-7 cells were transfected with pSG5-AR and FLAG-HBx, treated with 10 nM R1881 or ethanol, and fractionated into cytosolic and nuclear fractions. (*Upper*) Fifty micrograms of the extracts was loaded as lysate input for Western blot analysis by using antibodies for AR and HBx. IκBα antibody was used as the cytosolic protein control, and lamin B antibody was used as the nuclear protein control. (*Lower*) Two hundred fifty micrograms of lysates was used for immunoprecipitation and Western blot analysis by using AR and HBx antibodies.

significantly reduced with the ARΔHR construct (Fig. 2*E*, lane 5), the hinge region of AR was implicated in the HBx-enhanced activation of AR.

Interaction of AR and HBx Occurs in the Cytosolic but Not the Nuclear Compartment. To delineate the mechanism underlying HBx-enhanced AR activation, we first probed for any direct interaction between HBx and AR by coimmunoprecipitation. Cell lysates were prepared from Huh-7 cells cotransfected with FLAG-HBx and AR expression constructs. After immunoprecipitation with anti-FLAG antibody, the HBx-associated cellular complex was analyzed by Western blotting by using an anti-AR antibody to detect AR protein in the precipitate (Fig. 3*A*, lane 2). The results indicated that HBx and AR are associated in the same protein complex.

The region(s) required for AR to form a complex with the HBx was further investigated by using the four AR deletion constructs mentioned above for the coimmunoprecipitation analysis (Fig. 3*A*, lanes 3–6). Only the ARΔHBD construct could not be coimmunoprecipitated with HBx, indicating that the ligand-binding domain of AR was critical for its interaction with HBx (Fig. 3*A*, lane 6).

The subcellular location where HBx exerts its enhancement role was determined by fractionating the cell lysates into cytosolic and nuclear extracts. Whether in the presence of HBx, AR protein was mainly located in the cytosolic fraction in the absence of ligand and, as expected, a significant proportion of AR was translocated into the nucleus upon 10 nM R1881 treatment (Fig. 3*B Middle*, probed with AR antibody). However, HBx was mainly located in the cytosolic fraction, even in the presence of R1881 (Fig. 3*B Middle*, probed with HBx antibody). The subcellular distribution pattern of HBx and AR was also verified by immunofluorescent staining (data not shown). The AR present in the cytosolic fraction could be coimmunoprecipitated with HBx in both the presence or absence of R1881, and no interactions could be detected in the nuclear fraction (Fig. 3*B Bottom* of IP with anti-FLAG). We also used anti-AR antibody for the reciprocal immunoprecipitation analysis. Consistently, HBx could be detected in the AR-containing complex immunoprecipitated with lysates from the cytosolic fraction rather than from the nuclear fraction (data not shown). These results suggested that AR interacts with HBx mainly in the cytosolic region and in a ligand-independent manner.

c-Src Activity Is Involved in HBx-Enhanced AR Activation, Possibly by Affecting AR Phosphorylation. The above results indicated that HBx enhancement of AR reporter transactivation did not work by a physical association between HBx and AR, which should occur in the nucleus when the ligand is added. In addition, deletion analysis revealed that the HBD region of AR, which is responsible for its interaction with HBx (Fig. 3*A*), was not essential for the enhancing activity of HBx (Fig. 2*E*). These results lead to the possibility that a direct interaction between these two proteins is not the immediate mechanism for the enhancement of AR transactivation by HBx. Therefore, HBx might activate a specific cellular signaling pathway(s) in the cytosolic compartment that affects AR (wild type) and its transcriptional activity in a ligand-dependent manner.

The c-Src kinase-mediated signaling pathway attracted our attention for its known involvement both in HBx and AR signaling activities (32–34). To investigate the role of c-Src-mediated pathway in HBx enhanced AR activation, HepG2 cells cotransfected with AR and HBx constructs were treated with PP2, a relatively specific inhibitor of c-Src family kinases. The R1881-dependent reporter activity was found significantly reduced (Fig. 4*A*, lane 3). Because HBx-induced c-Src activation is mediated through increasing cellular calcium levels (32), we treated cells with cyclosporine A, an inhibitor for calcineurin. HBx-mediated androgen-response element activity was significantly suppressed (Fig. 4*A*, lane 4). Moreover, we included inhibitors for MEK, AKT, and JNK (U0126, LY294002, and SP600125, respectively) to test the possible involvement of a downstream transducer kinase for the resulting AR activation. MEK and AKT inhibitors could also suppress the activation effect (Fig. 4*A*, lanes 5 and 6); however, SP600125 did not have significant suppressive effect (Fig. 4*A*, lane 7). The inhibitory effect of cyclosporine A, PP2, U0126, and AKT indicated that a plausible axis of HBx triggered calcium elevation, c-Src activa-

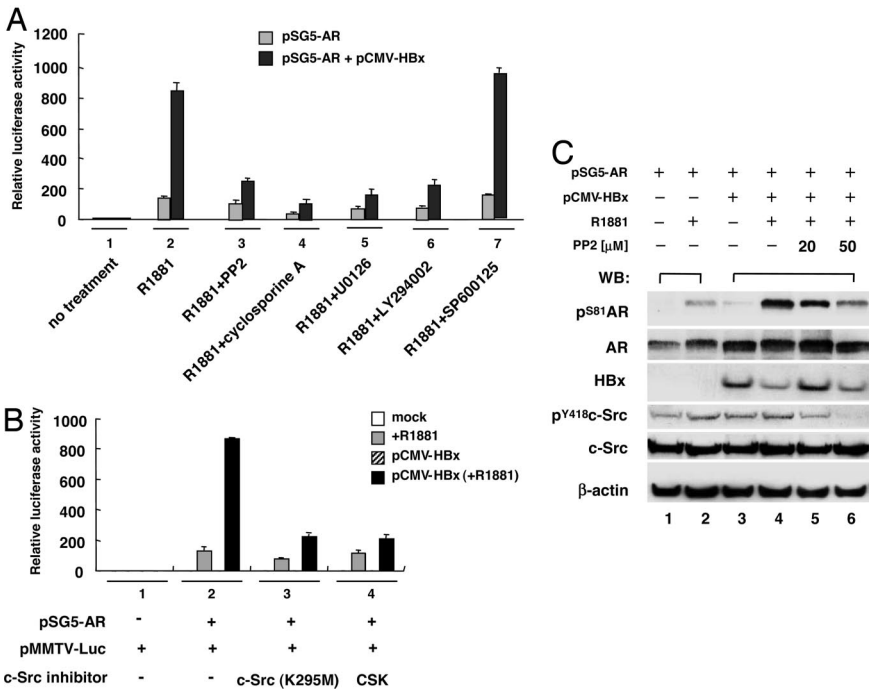

Fig. 4. Involvement of c-Src activity in HBx induced AR activation and AR phosphorylation. (*A*) HepG2 cells cotransfected with pSG5-AR, pCMV-HBx, pMMTV-luc, and pRL-CMV were untreated, treated with 10 nM R1881, or treated with a combination of the inhibitors PP2 (20 μM), U0126 (10 μM), LY294002 (100 nM), SP600125 (20 nM), and cyclosporine A (10 μg/ml), as indicated. (*B*) HepG2 cells were transfected with pSG5-AR, pCMV-HBx, pMMTV-Luc, pRL-CMV, and the dominant-negative c-Src mutant K295M or the CSK kinase expression plasmids, and treated with 10 nM R1881 as indicated. Luciferase activities are the average of three experiments (mean ± SD). (*C*) Huh-7 cells transfected with pSG5-AR and pCMV-HBx were treated with R1881 (10 nM) in combination with PP2 (20 or 50 μM). The cell lysates were harvested for Western blot analysis by using the indicated antibodies. The p-^{Y418}Src antibody was used as a control to verify the suppression of c-Src kinase activity by PP2 treatment.

tion, and downstream MEK and AKT activation might be involved in HBx-induced AR transcriptional activation.

To more specifically evaluate the role of c-Src activity in HBx-induced AR activation, we used two more specific c-Src inhibitors, the K295M c-Src kinase-dead dominant-negative mutant and the C-terminal c-Src kinase, in our analysis (35). In the presence of R1881, HBx-mediated AR activation was significantly inhibited by both constructs, and the reporter activity in cells transfected with AR alone was only slightly decreased (Fig. 4*B*, lanes 3 and 4). The results support a significant role of the c-Src signaling pathway in the HBx-induced AR activation.

To explore how c-Src signaling affects AR gene transactivation, we first investigated whether the signaling affected the phosphorylation of AR protein by using an antibody specific to the phosphorylated form of AR at Ser-81 (36). In the presence of R1881, AR was phosphorylated at a barely detectable level (Fig. 4*C*, lane 2, probed with antibody specific for P^{S81} of AR). However, when HBx was present together with R1881, the extent of AR phosphorylation increased 5- to 6-fold (Fig. 4*C*, lane 4). Although the amount of AR was noted as also increased in the presence of HBx, the increase of Ser-81 phosphorylation remained significant after the adjustment of the AR amount. Such an augmented phosphorylation was partially reduced when the cells were treated with PP2 in a dose-dependent manner (Fig. 4*C*, lanes 5 and 6). Therefore, HBx may affect AR through a c-Src-mediated modification of its phosphorylation status in a ligand-dependent manner.

Discussion

HBV-induced hepatocarcinogenesis is through a multistep process, accumulating the genetic events starting from the stage of liver cirrhosis, which accelerates an almost irreversible carcinogenic process (37). For effective prevention of HBV-related HCC, it is important to study both the host and viral factors predisposing hepatocytes to such a carcinogenic process. Because of the male preference of HCC, the androgen-signaling pathway has long been considered one of the important host factors involved in the carcinogenesis (4). For the viral factors, it is generally accepted that virus-induced chronic inflammatory necrosis might cause the hepatocytes to undergo proliferation and thus increase the occurrence of genetic aberrations. The finding that the male dominance of HBV-related HCC is much more significant than in HCV-related HCC prompted us to examine whether specific HBV viral factor(s) might also participate in male hepatocarcinogenesis by targeting to the AR signaling pathway.

Our study indicates that HBx is a key viral factor linking HBV infection and the increased risk of hepatocarcinogenesis in males. The synergistic cell-transforming effects of HBx and AR signaling were demonstrated by the enhancement of the anchorage-independent colony-forming capability in AML-12 cells. We further provide evidence for a coregulator role of HBx (even in the context of HBV genome) on AR-responsive gene expression, which has hitherto not been reported. The effect of HBx was also documented in the context of the HBV genome, which is presumably more likely reflecting the HBx amount corresponding with the natural HBV infection. Intriguingly, the HBx-mediated effect is androgen-concentration-responsive, increasing from 0.05 to 10 nM of DHT. The positive coregulator activity of HBx thus should be more evident in males than in females. Because the risk of HCC has been demonstrated to be significantly associated with higher androgen concentrations in male HBV carriers (19), higher androgen concentrations may combine with HBx to promote hepatocarcinogenesis in male HBV carriers, mainly through AR activation. We are currently testing this hypothesis in HBx-transgenic mice, in which most of the males develop HCC (6, 25). It is possible to knock out (or knock down) AR gene in these mice and then investigate the

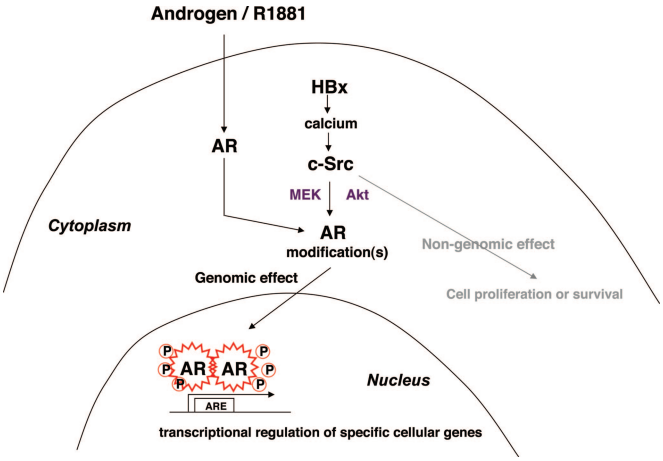

Fig. 5. A proposed model for HBx induced AR activation and carcinogenesis. HBx-mediated enhancement of AR activity is androgen-dependent and could be mediated through an indirect mechanism involving calcium and c-Src signaling pathways, which lead to subsequent augmentation of AR phosphorylation and increased transcriptional activities (the genomic effect). Alternatively, nongenomic effects mediated by c-Src signaling in the cytoplasm might affect cell proliferation and survival (indicated as gray characters), although the present study did not explore this possibility. The details of this proposed model are discussed in the text.

occurrence of HCC. It should provide conclusive genetic evidence for a role for AR in HBx-induced male HCC.

Because HBx mainly distributes in the cytosolic compartment irrespective of androgen treatment, it may activate AR by affecting preexisting cytosolic cellular signaling pathways. It has been established that HBx can stimulate various cytoplasmic signal transduction pathways, including Ras/MEK/MAPK, JNK/JAK/STAT, and phosphatidylinositol 3-kinase (PI3K)/Akt pathways (23, 24). This activity requires no direct interaction between HBx and any of these protein kinases. The c-Src kinase, an upstream activator of all these cytoplasmic signaling pathways, has been suggested to be the key switch turning on these kinase signaling cascades (23, 24). Bouchard *et al.* (32) demonstrated that HBx can activate c-Src indirectly by triggering the release of Ca^{2+} ions from the endoplasmic reticulum and mitochondria, which in turn activates the Ca^{2+}-responsive Pyk2 kinase and leads to c-Src activation. By showing that inhibitors of c-Src and calcium signaling can abrogate HBx-enhanced AR activation, our results supported the critical role of this axis in HBx-enhanced AR activation. Presently, an assessment of the signaling pathways downstream of c-Src activation has demonstrated that the enhancement of AR activity also decreases after treatment with inhibitors for MEK (U0126) and AKT (LY294002). The MEK/MAPK and PI3K/Akt downstream pathways are thus likely to be involved in HBx-enhanced AR activity. Based on our current results, we propose a model illustrating a possible pathway for HBx-enhanced AR activation (Fig. 5). However, in such a model, whether androgen-stimulated AR is involved in c-Src activation awaits clarification. Migliaccio *et al.* (33) reported that the N-terminal proline-rich stretch of AR could directly associate with the SH3 domain of c-Src and remove one of its intramolecular inhibitory interactions. In their study (33), the c-Src kinase can be further activated when a second inhibitory domain is disrupted after binding with activated estradiol receptor (ER) α (or β) through a phosphorylated tyrosine residue. Formation of the ternary complex (c-Src/AR/ER) can significantly increase c-Src activity (33).

The next issue to be addressed is the molecular mechanism(s) by which HBx enhances AR activity. Several posttranslational modifications of AR, including phosphorylation, acetylation, and sumoylation, profoundly affect its activity (38, 39). Because c-Src signaling might affect several downstream kinase signaling pathways, we thus first checked the effect of HBx on AR phosphorylation. Several phosphorylation sites on AR have been mapped, with the majority at serine residues. Phosphorylation at some of these sites is increased by androgen stimulation, such as at serines 16, 81, 256, 308, 424, and 650 (40). Some of these sites were identified as target sites of specific kinases, such as at serines 213 and 790 (phosphorylated by Akt) (41). By using antibody specific for the phosphorylated form of AR at Ser-81, we found that ligand-dependent AR phosphorylation at Ser-81 can be further up-regulated in the presence of HBx. Phosphorylation can also be partially blocked by PP2 treatment. These results implicate an HBx-induced calcium/c-Src pathway that affects AR posttranslational modification (Fig. 5). Because at least six serine residues of AR are phosphorylated upon R1881 treatment, Ser-81 phosphorylation status may represent only part of the response to HBx, and detailed analysis for HBx-induced changes in AR phosphorylation at individual sites (or some other unidentified sites) need to be investigated. The possibility that HBx may affect AR by other posttranslational modifications (acetylation or sumoylation) also needs to be clarified. Special attention should focus on modifications at the hinge region of AR, deletion of which affects HBx-enhanced AR activity.

Notably, our results demonstrate that HBx can enhance AR activity in the ARΔHBD construct even in the absence of ligand stimulation (Fig. 2*E*, lane 6), suggesting that HBx can also increase AR activity in a ligand-independent manner. However, our results showed that HBx alone does not increase the reporter activity for the wild-type AR in the absence of ligand. Therefore, HBx does not have ligand-independent AR activation activity. ARΔHBD is a mutant construct showing constitutive transactivation activity in a hormone-independent manner (31), but whose activity was still up-regulated by HBx. It identified the N terminus of AR as a target for the enhancing action of HBx. Because ARΔHBD is not the natural form of AR, the results help us identify the functional domains, not implying that wild-type AR acts similarly. It is possible that, once the conformation of AR changes after ligand binding, HBx might enhance AR in a ligand-independent manner, perhaps by affecting the posttranslational modification at its N terminus.

In addition to affecting the posttranslational modifications of AR, several other plausible mechanisms underlying HBx-induced AR activation also await investigation. For example, HBx might activate NF-κB activity, which may cross-talk with the androgen signaling pathway (42, 43). Second, HBx could affect proteosome activity and thus influence the stability of several cellular proteins, possibly including the AR gene (44). Third, by directly interacting with p53, HBx antagonizes the normal functions of p53, one of which is an inhibitor of AR activation (45, 46). Moreover, the cellular coregulatory proteins associated with AR might be changed in the presence of HBx. Whether these mechanisms are mediated through the c-Src activity is also worthy of study.

By soft agar colony-forming assay, our results clearly show the enhanced transforming potential of HBx in combination with AR. As noted, HBx was delivered into the cells by transient transfection, and its expression might not last for 3 weeks during the colony-formation assay. However, in natural viral infection, HBx is expressed transiently only in infected hepatocytes but not in cancers (23, 24, 29). Therefore, HBx likely behaves as an initiator to activate a sustaining carcinogenesis process. Concerning the carcinogenic mechanism, both genomic and nongenomic effects of AR might be involved in the carcinogenic process (Fig. 5; refs. 21 and 22). For the genomic effect, HBx-enhanced AR reporter activity was well documented in our study. The cellular target genes affected by such enhancement are currently being investigated by using microarray analysis. With regard to nongenomic effects, recent studies have demonstrated that cell growth-enhancing and antiapoptotic actions of androgen can be independent from its transcriptional activity

(47). In certain cells, the effect is mediated by c-Src activation of MAPK or Akt signaling pathways (21, 22). This cell transformation activity again converges to the HBx-mediated activation of c-Src. The carcinogenic effect concerning the nongenomic effect of AR becomes an important issue to be addressed.

Males are more susceptible than females to various diseases, including many infections (48, 49). However, the microbial factors for determining gender differences of susceptibility to diseases often remain unidentified. Using HBV-related HCC as an example, we have identified HBx as a previously undescribed class of noncellular coregulators of AR and a potential risk factor. These results might shed light on potential targets for future prevention or therapy for this type of HCC. Our results provide a plausible explanation for the male gender preference of HBV-related HCC. Furthermore, the study also provides a general mechanism for male vulnerability to microbe infections and subsequent cancer development in these individuals.

Methods

Plasmids, Chemicals, and Antibodies. The plasmid-expressing AR protein (pSG5-AR) and the AR-responsive androgen-response element-directed luciferase reporter plasmid (pMMTV-luc) (50) were kindly provided by Hsiu-Ming Shih (Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan). Four AR mutants, ARΔ1–188, ARΔ1–488, ARΔHR, and ARΔHBD, each with deletion of individual functional domains (31), were from Andrew C. B. Cato (Institute of Toxicology and Genetics, Karlsruhe, Germany). Plasmids expressing the wild-type HBx gene (pCMV-HBx) and several function-impaired mutants ($HBx_{7C->7S}$, $HBx_{61C->61S}$, $HBx_{69C->69S}$, and $HBx_{90P,\ 91K->90V,\ 91L}$) (30) were obtained from Shin-Lian Doong (Department of Microbiology, National Taiwan University College of Medicine, Taipei, Taiwan). Two human c-Src inhibitors, the C-terminal c-Src kinase (CSK) and the c-Src dominant-negative mutant (K295M) (35), were gifts from Ching-Chow Chen (Institute of Pharmacology, National Taiwan University College of Medicine). A plasmid expressing full-length HBV and able to initiate replication, pHBV-48 (29), was from Hui-Lin Wu (Hepatitis Research Center, National Taiwan University Hospital, Taipei, Taiwan). Using this construct, we have further constructed plasmid pHBV48-X_0 with an HBx gene knockout. It was constructed by site-directed mutagenesis to create a non-sense stop codon (C22A) within the HBx gene (51) by using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The oligonucleotide 5′-TGGCTGCTAGGCTGTACTGCTAACTGGATCCTTCGCGGGAC-3′ and its corresponding reversed oligonucleotide were used as primers for the reaction.

To establish a cell line to stably expressing AR, we modified the AR expression plasmid by inserting a puromycin-selection marker. The plasmid pSG5-AR-puro was constructed by inserting the SmaI/EcoRI fragment from pSUPER-retro plasmid (52), which contains a PGK promoter and a puromycin-resistance gene, into the NdeI site of pSG5-AR.

Chemicals were purchased from commercial suppliers. The AR agonist R1881 was from Perkin-Elmer (Wellesley, MA), the androgen 5α-DHT (5α-androstan-17β-ol-3-one) was from Fluka (Buchs, Switzerland), and cyclosporine A was from Sigma-Aldrich (St. Louis, MO). Specific kinase inhibitors for c-Src, MEK, AKT, and JNK used in this study were PP2, U0126, LY294002, and SP600125, respectively (Calbiochem, San Diego, CA). The reagents were prepared by dissolving in ethanol or DMSO and were added to the cultured cells with the proper dilutions.

Antibodies to FLAG (M2) and β-actin were obtained from Sigma-Aldrich. Antibodies to AR (C19 and N20) were acquired from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-P^{S81}-AR and anti-P^{Y418}-Src antibodies were purchased from Upstate Biotechnology (Charlottesville, VA) and BioSource Europe (Nivelles, Belgium), respectively. Polyclonal HBc and HBx antibodies were generated in our laboratory by immunizing a New Zealand rabbit (10–12 weeks old) with recombinant His-tag fusion protein expressed from *Escherichia coli* BL21 (DE3) pLysS.

Cell Culture, Transfections, and Luciferase Reporter Assay. HepG2 and Huh-7 cells were cultured in DMEM (Gibco, Carlsbad, CA) supplemented with 10% FBS (HyClone, South Logan, UT); AML12 cells were cultured in DMEM/F-12 medium with 10% FBS, including a mixture of insulin, transferrin, and selenium (Gibco), and were kept in a 5% CO_2 incubator at 37°C. The transfection experiments were conducted by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), following the manufacturer's instructions. In brief, 4 μg of a plasmid DNA mixture was used for each transfection reaction for one well of a six-well plate, containing 3.5×10^5 Huh-7 or AML12 cells and 5×10^5 HepG2 cells. The pCDNA3 vector plasmid was used to adjust the amount of total transfected DNA to be equivalent.

At 4 h before transfection, cells were replenished with fresh DMEM containing 5% (vol/vol) dextran-coated charcoal-stripped FBS (CT-FBS). After transfection, cell cultivation continued in the CT-FBS supplemented medium with or without addition of DHT or R1881. The cell lysates were harvested 24 h after treatment. For determination of the MMTV-luc reporter activity, we included the pRL-CMV plasmid (Promega, Madison, WI) as an internal standard for normalization of transfection efficiency. An equal protein amount of the lysate was used for determination of the enzyme activity of firefly and *Renilla* luciferase following the manufacturer's instructions (Promega).

Protein Extraction, Cellular Fractionation, Immunoprecipitation, and Western Blot Analysis. Total cellular protein was extracted by lysing cells with the buffer (20 mM Hepes, pH 7.9/0.2 M NaCl/1 mM EDTA/1 mM EGTA/0.2% Triton X-100) containing 1 mM PMSF and complete protease inhibitor mixture (Roche, Mannheim, Germany). We used the ProteoExtract Subcellular Proteome Extraction Kit to separate the cytosol and nucleus fractions following the manufacturer's instructions (Calbiochem, Darmstadt, Germany). The lysate (250 μg of protein) was used for immunoprecipitation and the subsequent Western blot analysis by following the protocols described (50).

Soft-Agar Anchorage-Independent Colony-Forming Assay. Cells (1×10^4) were seeded in 60-mm soft-agar plates in the presence or absence of 5 nM R1881. Cells were replenished with CT-FBS DMEM/F-12, R1881 supplemented medium every 3 days. After 21–28 days, the plates were stained with 0.3% crystal violet, and the colonies were counted by microscopic observation (53). Each assay was conducted in duplicate.

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